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ORIGINAL PAPER

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Homologous recombination and double-strand break repair in the transformation of *Rhizopus oryzae*

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Abstract Genetic transformation of the Mucorales fungi has been problematic, since DNA transformed into the host rarely integrates and usually is mitotically unstable in the absence of selective pressure. In this study, transformation of *Rhizopus oryzae* was investigated to determine if the fate of introduced DNA could be predicted based on double-strand break repair and recombination mechanisms found in other fungi. A transformation system was developed with uracil auxotrophs of *Rhizopus oryzae* that could be complemented with the *pyrG* gene isolated in this work. DNA transformed as circular plasmids was maintained extrachromosomally in high-molecular-weight (> 23 kb) concatenated arrangement. Type-I crossover integration into the *pyrG* locus and type-III *pyrG* gene replacement events occurred in approximately 1–5% of transformants. Linearization of the plasmid pPyr225 with a single restriction enzyme that cleaves within the vector sequence almost always resulted in isolates with replicating concatenated plasmids that had been repaired by end-joining recombination that restored the restriction site. The addition of a 40-bp direct repeat on either side of this cleavage site led to repair by homologous recombination between the repeated sequences on the plasmid, resulting in loss of the restriction site. When plasmid pPyr225 was digested with two different enzymes that cleave within the vector sequence to release the *pyrG* containing fragment, only *pyrG* gene replacement recombination occurred in transformants. Linearization of plasmid pPyr225 within the *pyrG* gene itself gave the highest percentage (20%) of type-I integration at the *pyrG* locus. However, end-joining repair and gene replacement events were still the predominant types of recombination found in transformations with this plasmid topology.

Keywords *Rhizopus* · Transformation · Recombination · Mucorales · Zygomycota

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Introduction

Rhizopus is a filamentous fungus with a history of use in the production of fermented foods, industrial enzymes (e.g., glucoamylase and lipase), organic acids (e.g., lactate and fumarate) and corticosteroids; all the while having the unfortunate reputation of being a food spoilage organism, a plant pathogen and an opportunistic human pathogen. Despite its importance, the techniques for genetic manipulation of this remarkable organism are still in an early stage of development compared to those used for many other fungi (for review, see Fincham 1989). In general, this holds for all fungi in the order Mucorales (class Zygomycetes), mainly due to our lack of understanding of the recombination and replication mechanisms that affect the fate of introduced DNA in these species.

Genetic transformation systems have been developed for a small number of Mucorales fungi, such as *R. delemar* (Horiuchi et al. 1995), *R. niveus* (Yanai et al. 1990, 1991; Liou et al. 1992; Takaya et al. 1996), *Mucor circinelloides* (van Heeswijck and Roncero 1984; Anaya and Roncero 1991; Arnau et al. 1991; Benito et al. 1992, 1995; Iturriaga et al. 1992; Arnau and Stroman 1993), *Absidia glauca* (Wöstemeyer et al. 1987; Burmester et al. 1990, 1992), *Phycomyces blakesleeanus* (Revuelta and Jayaram 1986; Arnau et al. 1988; Suarez and Eslava 1988) and *Rhizomucor pusillus* (Wada et al. 1996). Unfortunately, all of these systems have problems associated with mitotic instability of the introduced DNA and/

or lack the ability to target integration into specific loci on the chromosomes. This is because DNA transformed into Mucorales fungi rarely integrates, as is common with most fungi, but is thought to replicate extrachromosomally. Autonomous replication of DNA typically requires that the introduced DNA contain sequences for initiation of replication and an origin of replication. The *Saccharomyces cerevisiae* 2 μm autonomous replicating sequence (ARS) is perhaps the classic example of fungal extrachromosomal DNA replication (Beggs 1978). Since the 2 μm origin of replication does not function effectively in other fungi, researchers identified similar sequences for other fungi that confer autonomous replication with varying degrees of success (Das et al. 1984; Gems et al. 1991). In Mucorales fungi (van Heeswijck 1986; Benito et al. 1995), as well as some members of the Basidiomycetes (Edman and Kwon-Chung 1990; Fotheringham and Holloman 1990), introduced DNA replicated autonomously without the addition of any known sequences that confer initiation of replication. It is not clear whether the requirements for such replication sequences are less stringent or if they simply occur more often in these fungi. Attempts to isolate sequences that improve the efficiency of stable replication in Mucorales fungi have met with some success (Suarez and Eslava 1988; Roncero et al. 1989; Burmester et al. 1992).

Integration into the genome is the ideal method of ensuring stable replication of the introduced DNA, and is required for disruption of specific genes. However, integration has been problematic with Mucorales fungi, and reports of integration are poorly understood or not well substantiated by hybridization data (Arnau et al. 1988, 1991; Burmester et al. 1990; Yanai et al. 1990; Arnau and Stroman 1993; Horiuchi et al. 1995; Wada et al. 1996). Erroneous assumptions are often made about integration, since DNA transformed into Mucorales fungi appears to replicate in a concatenated high-molecular-weight form (Gonzalez-Hernandez et al. 1997; this study) that co-migrates with genomic DNA. To date, no controlled transformation study has been performed with Mucorales fungi to examine the recombination and repair events that mediate integration of introduced DNA.

The goal of this work was to find methods of directing genomic integration into the Mucorales fungus *Rhizopus* by using linearized transforming DNA. It was assumed that methods of recombination would not differ from models of double-stranded DNA repair and integration for *S. cerevisiae* (for reviews, see Paques and Haber 1999; Lewis and Resnick 2000), but only be complicated by the tendency of DNA to replicate autonomously. Evidence is provided that clearly demonstrates that linearized DNA is typically repaired by illegitimate end-joining or homologous plasmid recombination (i.e., if sufficient regions of duplicate homology are present). Furthermore, homologous chromosomal integration and gene replacement can occur independently or in combination with either of the above types of repair events.

Materials and methods

Isolation of the *R. oryzae* OMP decarboxylase gene (*pyrG*)

R. oryzae (syn. *R. arrhizus*) NRRL 395 was the source strain for the isolation of the orotidine monophosphate (OMP) decarboxylase auxotrophs and the complementing *pyrG* gene. The *pyrG* gene was isolated by first comparing genes coding for the OMP-decarboxylase proteins from *R. niveus* (Horiuchi et al. 1995), *M. circinelloides* (Benito et al. 1992) and *P. blakesleeanus* (Diaz-Minguez et al. 1990) to find conserved regions. These sequences were then used to develop the degenerate oligonucleotide primers 5′-ATTGAYAT-TGTKGAAGACTTYGA-3′ and 5′-CCACTCTCMACAATNA-CTTC-3′ for PCR of *R. oryzae* genomic DNA. Conditions were as described by Ausubel et al. (1995), except that the following program was used: 30 cycles of 95°C for 45 s, 55°C for 60 s, and 72°C for 90 s. An amplified product of 544 bp was determined to be similar to other *pyr*G or equivalent OMP decarboxylase-encoding genes (e.g., *URA3*) and was used as a probe to isolate hybridizing clones from a genomic library previously described (Skory 2000).

Introns were identified by PCR amplification and sequencing of cDNA. RNA isolated by a hot-phenol method (Ausubel et al. 1995) from a culture grown in RZ minimal medium (Skory 2000) served as the template for cDNA synthesis using the SuperScript Preamplification System for First Strand Synthesis (Invitrogen, Carlsbad, Calif.). Multiple primer combinations were used to amplify overlapping fragments spanning the entire *pyrG* coding region. Sequence information from these fragments was then compared to that obtained from genomic sequence to identity the positions of introns.

Isolation of *R. oryzae* uracil auxotrophs

Germinating spores were mutated with 1-methyl-3-nitro-1-nitrosoguanidine (NNG) as previously described (Skory et al. 1998) and then distributed onto 18 potato dextrose agar (Difco/Becton Dickinson, Sparks, Md.) plates containing 0.5 mg uracil/ml. Multiple plates were used to ensure the isolation of independent mutation events, and sporulation under non-selective conditions allowed segregation of mutant alleles. Selection for uracil auxotrophs was performed by transferring spores from each plate to RZ medium (Skory 2000) supplemented with 2.5 mg 5-fluoroorotic acid (FOA)/ml and 0.5 mg uracil/ml. FOA is toxic to uracil prototrophic cells, due to the formation of the nucleotide analog 5-fluorouracil (Boeke et al. 1987). Spores harvested from each FOA-containing plate were diluted in sterile water to allow separation of single-spore isolates. Individual isolates were then tested for uracil auxotrophy by examining growth on RZ medium with and without 0.5 mg uracil/ml. All incubations of *R. oryzae* were performed at 30°C.

Enzymatic characterization of uracil auxotrophs

Mutations in either of two enzymatic steps, conversion of orotic acid to OMP and of OMP to uridine monophosphate (UMP), can lead to FOA resistance. Differentiation between strains deficient in OMP pyrophosphorylase (encoded by the *pyrF* gene) and OMP decarboxylase (encoded by the *pyrG* gene) was accomplished using ^{14}C labeling to examine each conversion step. Enzymatic analyses were as described by Skory et al. (1990). Briefly, cell-free protein extracts from each of the FOA-resistant mutants were incubated with either [^{14}C]orotic acid plus 5-phophoribosyl pyrophosphate or ^{14}C[OMP]. Reactions were stopped after the appropriate time and then analyzed by TLC to identify the labeled OMP and/or UMP.

Identification of the *pyrG* mutation in the host strain used for transformation

Genomic DNA from *R. oryzae* isolate PYR-17, one of the uracil auxotrophs confirmed to be deficient in OMP decarboxylase was

used as the template for PCR amplification and sequencing of the *pyrG* locus. Multiple primer combinations were used to amplify overlapping fragments spanning the entire *pyrG* coding region, as well as 632 bp of upstream and 22 bp of downstream-untranslated region. Sequences from these fragments were then compared to that obtained from the original *R. oryzae* NRRL 395 genomic sequence to identify possible mutations.

Design and introduction of transformation vectors into the host strain

A 2.25-kb *Eco*RI fragment (Accession No. AF497632) from a partially digested *pyrG*-containing isolate from the genomic library, was subcloned into the *Eco*RI site of pBluescript II KS- (Stratagene, La Jolla, Calif.). The resulting plasmid, pPyr225, can be linearized with *Bam*HI or double-digested with *Bam*HI and *Kpn*I to release the *pyrG* gene fragment (Fig. 1). Plasmid pPyr225 was further modified by introducing a 40-bp direct repeat of the polylinker region, made from complementary oligonucleotides, between the *Bam*HI and *Xba*I sites (Fig. 2). Linearization of this latter plasmid, pPLDR, with *Bam*HI results in a fragment with a repeat at each end. Plasmids digested with restriction enzymes prior to fungal transformation were gel purified two times or more, until purified DNA was no longer capable of transforming *E. coli* XL1-Blue MRF' cells (Stratagene) which had a transformation competence of greater than 1×10^8 tranformants/μg for supercoiled pUC19 monomers. Vector sequence released by digestion with both enzymes was removed from the *pyrG* gene fragment during gel purifications.

Transformations of the *R. oryzae pyr*G mutant were performed by microprojectile particle bombardment (BioRad, Hercules, Calif.). Optimal conditions for transformation were achieved using tungsten (M5) particles for DNA delivery and 1100-psi (7.6 MPa) rupture discs. The distance between the rupture disk and the tungsten particle carrier was approximately 1 cm, and the distance between the launch assembly and the target was 8 cm. DNA was coated onto the tungsten particles according to the manufacturer's recommendations. Ungerminated spores were transformed directly on RZ minimal medium plates, since no recovery time for auxotrophic selection was required. Approximately 5–7 days after bombardment, spores were collected and diluted in sterile water to obtain single-spore isolates. Only one isolate per plate was used for further analysis to avoid multiple progeny originating from the same transformation event.

Molecular analyses

Southern analyses were performed using the Genius system (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's recommendations. Digoxigenin-labeled λ DNA, cleaved with *Hin*dIII, was included in the protocol to serve as a DNA molecular weight marker on the filters. The probe used for analyses of transformants was prepared by PCR with the primers PP1 (5′-ATAGCGAGCGTGCCAAACAAC-3′) and PP2 (5′-TTCAAGATATGCGTCCCAACCA-3′) for PCR amplification and labeling of an internal 848-nt region of the *pyrG* region. The region around the *Bam*HI site used for linearization of plasmids pPyr225 and pPLDR was sequenced in transformants by first using total DNA to amplify the polylinker region with the primers PP3 (5′-AGGGTCTTGCCGAGGTTGGTCTTC-3′), which anneals within the *pyrG*, and BSII (5′-CCGATTTCGGCCTATTGGTTA-3′), which anneals within the pBluescript II vector. Sequencing of these amplified fragments was then performed with the M13 (-20) forward primer and PP4 (5′-AACAAGCCCAAAGATACCGTCAAG-3′).

Results

Isolation of the *R. oryzae pyrG* gene

Several overlapping fragments that hybridized with the PCR-amplified *pyrG* probe were isolated from the genomic library. Through subcloning and sequencing, the coding region of the *pyrG* gene was localized to a

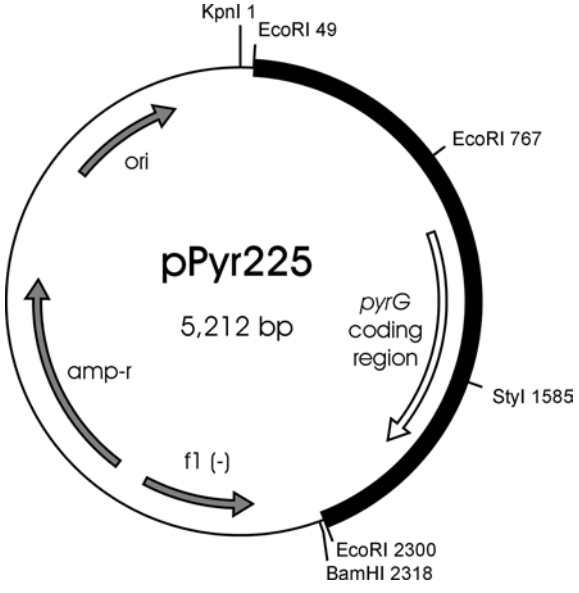

Fig. 1 Restriction map of the plasmid pPyr225 used for transformation studies. The *filled box* represents a 2.25-kb *Eco*RI genomic fragment from *R. oryzae* that was cloned into the *Eco*RI site of the vector pBluescript II KS-. The *pyrG* coding region contained on this plasmid is indicated by the *open arrowz*

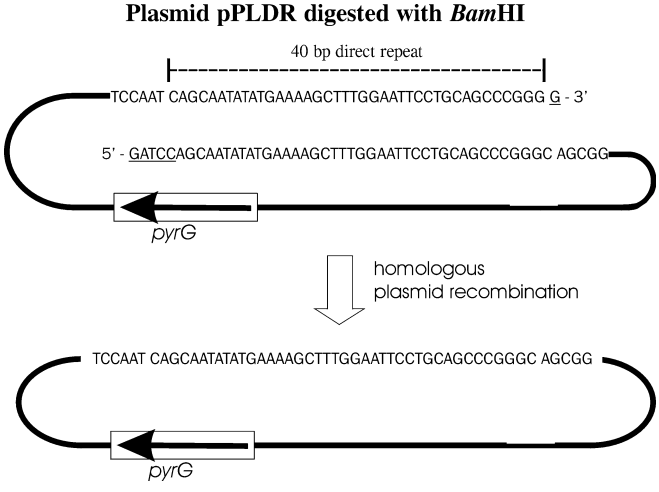

Fig. 2 Homologous recombination between 40-bp direct repeats on plasmid pPLDR (polylinker direct repeat). A 40-bp direct repeat of the polylinker region was incorporated into plasmid pPyr225, such that linearization with *Bam*HI results in one repeat on either side of the double-strand break. Only the top strands of the direct repeats are shown and the single-strand overhangs resulting from digestion with *Bam*HI are *underlined*. The sequence of this region following homologous recombination or crossover has been confirmed. Representation of this recombination as an intramolecular event instead of an intermolecular event is conjectural

1.5-kb *Eco*RI fragment (Accession No. AF497632). The positions of start/stop codons were inferred from sequence comparisons with other *pyr* genes (Diaz-Minguez et al. 1990; Benito et al. 1992; Horiuchi et al. 1995) and resulted in the identification of a 911-nt coding region. The coding region was 98% identical, and 5′ and 3′ untranslated sequences closer to 90% identical, to the corresponding segments of the *R. niveus pyr4* gene (Horiuchi et al. 1995). Two introns, 56 and 58 bp in length, were identified by sequence comparisons with amplified cDNA. Processing of the introns should result in a putative 230-amino acid protein with 100% identity to the *R. niveus* OMP decarboxylase protein.

Isolation and analysis of *R. oryzae* uracil auxotrophs

Mutated cultures that were able to grow and sporulate on FOA-containing medium were purified by single-spore isolation. Only one isolate per plate, from the original 18 FOA-containing plates, was used for further study. All isolates except one were able to grow in the presence of FOA. Transfers of these spores onto minimal medium with and without uracil revealed that all 17 of the cultures were uracil auxotrophs.

We initially tried to analyze OMP decarboxylase activity using spectrophotometric methods (Schwartz and Neuhard 1975). However, inconclusive data led us to use ^{14}C analysis to examine the conversions. Enzymatic ^{14}C analyses showed that five of the 17 uracil auxotrophs were deficient in OMP decarboxylase activity, encoded by *pyrG*, while still having a functional OMP pyrophosphorylase. *R. oryzae* NRRL 395 served as a control to demonstrate the ability to complete both conversion steps (data not shown). One of the *pyrG* mutants, PYR-17, was chosen for transformation after determining that it had a reversion frequency of $<10^{-9}$ per spore. With this mutant, no detectable germination or spore swelling occurred on minimal medium lacking uracil.

Sequence analyses of overlapping fragments obtained by PCR amplification of the *pyrG* gene from the *R. oryzae* mutant PYR-17 revealed only a single nucleotide change from the original genomic sequence. The mutation was a G→A transversion that occurred 181 bp downstream of the start codon, at the 5′ end of the splice junction for the first intron. It is assumed that this change would prevent the intron from being removed and thus result in a frame shift and premature termination of the protein.

Transformation with covalently closed pPyr225

Biolistic transformation with circular pPyr225 plasmids consistently yielded numerous prototrophic colonies that appeared within 3–4 days. No effort was made to calculate transformation efficiency, since only one transformant per plate was used for further testing. However, it is estimated that the transformation frequency was approximately 10–50 transformants per μg DNA, depending on various factors such as humidity (which affects clumping of tungsten particles) and density of recipient spores (higher spore concentrations increase the likelihood of transformation). The rate of growth for transformants transferred as mycelial plugs to new minimal medium was comparable to that of the original *R. oryzae* NRRL 395 parent strain. However, rates of transfer of the prototrophic phenotype to the next spore generation were very inconsistent. The frequency of prototrophic single spore-derived colonies varied from 0.1 to 100% when spores were harvested from transformants grown on selective medium. Repeated serial transfer of each spore generation on complex or uracil-containing medium almost always resulted in complete reversion to auxotrophy.

Southern analysis of a typical isolate transformed with circular pPyr225 demonstrated that the plasmid was not integrated into genomic DNA, but was replicating autonomously in a high-molecular-weight form (Fig. 3). Hybridization patterns using a *pyrG* gene fragment as probe showed that uncut plasmid DNA comigrated with genomic DNA, even though electrophoresis was performed in the presence of ethidium bromide to induce supercoiling of the circular plasmids that might be present. The large plasmid form present in uncleaved transformant DNA was reduced to the size of the original pPyr225 plasmid (5.2 kb) by linearization with *Bam*HI, although a 10.4-kb hybridizing-band was detected and is believed to be a linear dimer resulting from incomplete digestion of the concatenated plasmid.

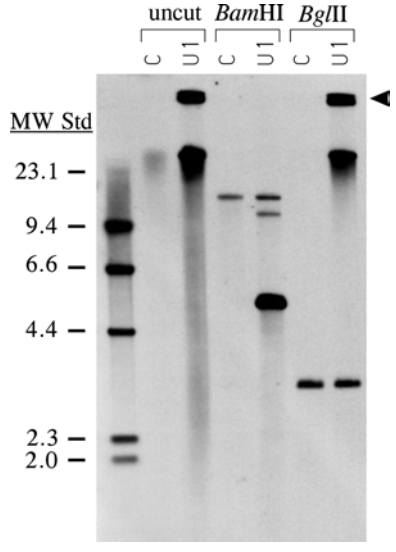

Fig. 3 Southern hybridization of DNA from a representative isolate of *R. oryzae* (U1) transformed with uncut plasmid pPyr225. Genomic DNA from untransformed *R. oryzae* (C) served as a control. Restriction enzymes used to digest total DNA for Southern analysis are shown *above* isolate names. An internal region of the *pyrG* coding region was used as the probe. Labeled *Hin*dIII fragments of λ DNA were used as molecular weight standards, and their sizes are shown (in kb) on the *left*. The *arrow* on the *right* marks the location of the wells

The genomic *pyrG* fragment hybridized at approximately 15 kb in both control and transformant *Bam*HI-digested DNA. The lack of integration into the *pyrG* locus is confirmed by the absence of a shift in the genomic 3.2-kb *pyrG* fragment following digestion with *Bgl*II, which does not cut within plasmid pPyr225.

The *pyrG* hybridization signal strengths for the replicating plasmids are considerably higher than that from the genomic *pyrG*, presumably because multiple copies of the plasmid are present per nucleus. This copy number was not consistent and ranged from only a few copies per nucleus to well over 25 (data not shown). Plasmid was recovered from 20 *Rhizopus* transformants by electroporation of undigested DNA into *E. coli* and selection for ampicillin resistance. No detectable differences in restriction digestion patterns could be found in isolated plasmids, suggesting that no major rearrangements had occurred.

Analysis of more than 50 isolates confirmed that integration of circular plasmid was rare, accounting for approximately 1–5% of transformants. When integration did occur, it was always by type-I homologous recombination (Hinnen et al. 1978) into the *pyrG* locus (data not shown). This type of recombination is usually described as single cross-over integration between the plasmid and genomic regions of homology, thus resulting in two copies of the homologous region. In this case, one copy of the *pyrG* gene is assumed to be non-functional due to the *pyrG-181* mutation. Type-I integration is often referred to as homologous additive integration. These transformants were found to be mitotically stable: no change in phenotype or Southern hybridization patterns was observed following several spore generations of non-selective growth. Type-III gene replacement of the *pyrG* also occurred in a similar percentage of transformants and was indicated by Southern hybridization patterns that did not differ from those of untransformed controls (data not shown). This type of integration or repair of the chromosomal mutation requires crossing over on both sides of the chromosomal mutation and excludes integration of non-homologous plasmid DNA (i.e., vector sequence).

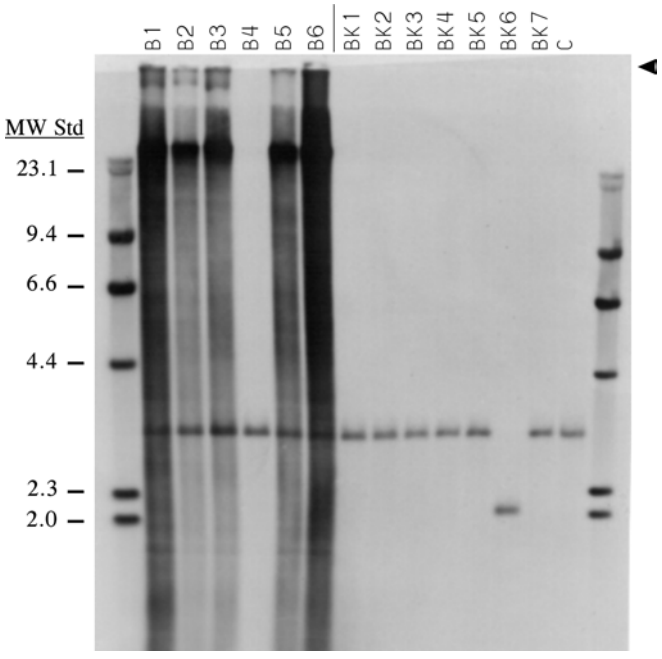

Fig. 4 Southern hybridization of *Bgl*II-digested DNA from *R. oryzae* isolates transformed with *Bam*HI-linearized pPyr225 (isolates B1–B6) or *Bam*HI + *Kpn*I-linearized pPyr225 (isolates BK1-BK7). DNA from untransformed *R. oryzae* (C) served as the control. An internal region of the *pyrG* coding region was used as probe. Labeled *Hin*dIII fragments of λ DNA were used as molecular weight standards, and their sizes are shown (in kb) on the *left*. The *arrow* on the *right* marks the location of the wells

Transformation with pPyr225 linearized outside of *pyrG*

Prior to transformation, plasmid pPyr225 was digested with either *Bam*HI or a combination of *Bam*HI and *Kpn*I. These sites are located within the polylinker region of the pBluescript plasmids and border each side of the *pyrG* fragment. Six isolates from the transformation with *Bam*HI-linearized DNA were analyzed by Southern analysis after digestion with *Bgl*II, which does not cleave plasmid pPyr225 (Fig. 4; isolates B1–B6). There was no evidence of integration into the *pyrG* locus, as indicated by the absence of a shift in the 3.2-kb genomic *pyrG* fragment. All but one of the transformants, B4, appeared to have plasmids that were replicating autonomously in a high-molecular-weight arrangement. We are confident that these isolates did not arise from transformation with undigested circular plasmid, since linearity of DNA was always confirmed by the absence of ampicillin-resistant cells in *E. coli* transformations. Plasmid DNA in transformants with replicating concatenates could be reduced to the size of the original plasmid by *Bam*HI digestion, demonstrating that this restriction site was restored by some type of end-joining recombination event (Fig. 5; isolates B1–B6, excluding B4). A 10.4-kb band, as seen with *Bam*HI digestion in Fig. 3, was present and is likely to represent a linear dimer resulting from incomplete digestion of the plasmid DNA. However, we cannot exclude the possibility that this presumed dimer arose from the occasional loss of the *Bam*HI site as a result of recombination. PCR amplification and sequencing of this plasmid region showed a complete *Bam*HI recognition sequence for transformants (data not shown). Isolate B4 did not differ in hybridization pattern from the control and is likely to represent an instance of type-III repair of the mutation in the genomic *pyrG* gene. Southern hybridizations with vector sequence as probe did not result in detection of any signal for this isolate (data not shown).

Transformation with the 2.3-kb *Bam*HI-*Kpn*I *pyrG* fragment resulted in type-III gene replacement events in all but one, BK6, of the seven transformants examined (Fig. 4; isolates BK1–BK7). Transformant BK6 was the

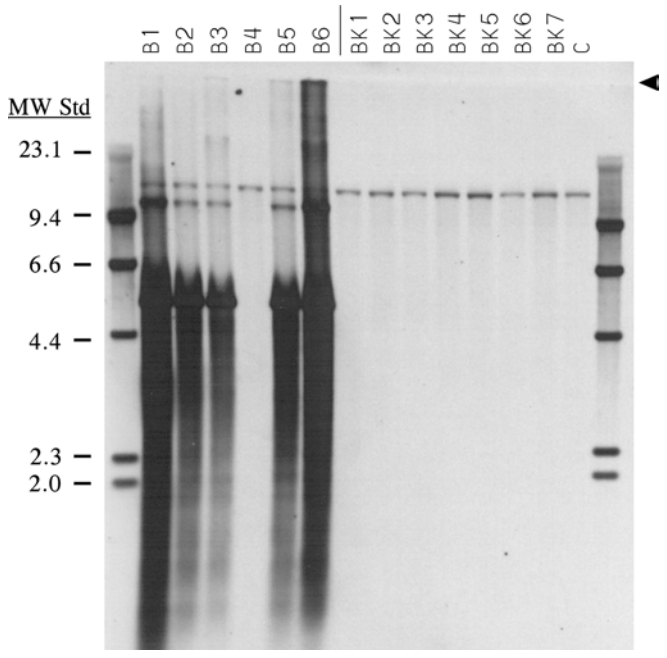

Fig. 5 Southern hybridization of *Bam*HI-digested DNA from *R. oryzae* isolates transformed with *Bam*HI-linearized pPyr225 (isolates B1–B6) or *Bam*HI + *Kpn*I-linearized pPyr225 (isolates BK1–BK7). DNA from untransformed *R. oryzae* served as control (C). An internal region of the *pyrG* coding region was used as probe. Labeled *Hin*dIII fragments of λ DNA were used as molecular weight standards, and their sizes are shown (in kb) on the *left*. The *arrow* on the *right* marks the location of the wells

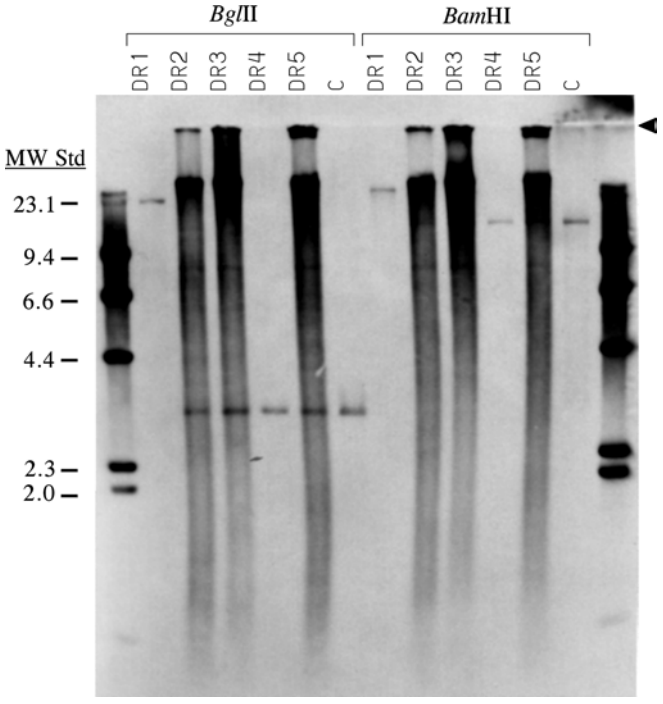

Fig. 6 Southern hybridization of DNA from *R. oryzae* isolates (DR1-DR5) transformed with *Bam*HI-linearized plasmid pPLDR. DNA from untransformed *R. oryzae* served as the control (C). Restriction enzymes used to digest total DNA for Southern analysis are shown *above* isolate names. An internal region of the *pyrG* coding region was used as probe. Labeled *Hin*dIII fragments of λ DNA were used as molecular weight standards, and their sizes are shown (in kb) on the *left*. The *arrow* on the *right* marks the location of the wells

only isolate that differed from the control in *Bgl*II digests. The expected signal at 3.2 kb was shifted to approximately 2 kb, suggesting that a partial deletion of the genomic *pyrG* had occurred. This shift was not evident when Southern analysis was performed with *Bam*HI-digested DNA (Fig. 5; isolates BK1–B7).

Effect of direct repeats flanking the *Bam*HI linearization site

Direct repeats from the polylinker region were introduced in such a way that digestion of the resulting plasmid pPLDR with *Bam*HI resulted in a 40-bp direct repeats bordering each side of the cleavage site (Fig. 2). Southern analysis of five isolates obtained from transformation with *Bam*HI-linearized pPLDR revealed unique recombination events. Hybridization patterns with *Bgl*II, which does not cleave the plasmid, clearly demonstrated that transformant DR1 contains an integrated tandem array comprising four copies of the plasmid, as evidenced by the shift of the *pyrG* fragment from 3.2 kb to approximately 24 kb (Fig. 6; *Bgl*II digestion). Transformant DR4 showed no difference in hybridization pattern from the control, and was assumed to result from a type-III *pyrG* gene replacement event. The remaining isolates DR2, DR3 and DR5 appeared to have plasmid DNA replicating autonomously in a concatenated arrangement. Unlike previous transformations with *Bam*HI-linearized plasmids, plasmid sequences from pPLDR transformants could not be digested with *Bam*HI (Fig. 6; *Bam*HI digestion). Furthermore, even integrated pPLDR DNA from isolate DR1 lacked a *Bam*HI recognition sequence, indicating that plasmid recombination had probably occurred prior to integration. PCR amplification and sequencing of plasmid DNA from transformants DR1, DR2, DR3 and DR5 confirmed that the *Bam*HI site was lost through homologous recombination between the direct repeats (Fig. 2).

Transformation with pPyr225 linearized within *pyrG*

In an effort to more efficiently direct integration into the *pyrG* locus, plasmid pPyr225 was linearized with *Sty*I, which cuts within the coding region of *pyrG*. At least four different types of recombination events were apparent from Southern analysis of *Bgl*II-digested DNA from five tranformants (Fig. 7). A single-copy integration of the 5.2-kb plasmid into the *pyrG* locus had occurred in transformant S1, as indicated by the shift of the *pyrG* fragment from 3.2 kb to 8.4 kb. The *pyrG* signal in transformant S5 also shifted, but appeared to derive from at least four copies in tandem, resulting in a

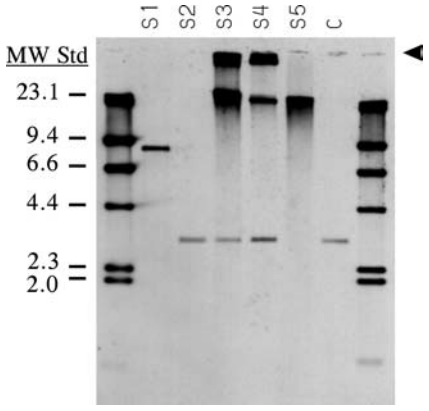

Fig. 7 Southern hybridization of *Bgl*II-digested DNA from *R. oryzae* isolates (S1–S5) transformed with *Sty*I-linearized plasmid pPyr225. DNA from untransformed *R. oryzae* served as the control (C). Restriction enzymes used to digest total DNA for Southern analysis are shown *above* isolate names. An internal region of the *pyrG* coding region was used as probe. Labeled *Hin*dIII fragments of λ DNA were used as molecular weight standards, and their sizes are shown (in kb) on the *left*. The *arrow* on the *right* marks the location of the wells

MW shift of over 20 kb. The intensity of the hybridizing band was greater than that expected from four copies, and this suggested that this transformant may also possess plasmids that are replicating autonomously in a concatenated arrangement. Transformant S2 showed no difference in hybridization pattern from the control and probably represents a gene replacement of the *pyrG* mutation. The last two transformants, S3 and S4, contained concatenated plasmids that are replicating autonomously in a high-molecular-weight form.

Discussion

This first description of a transformation system for *R. oryzae* provides the most comprehensive demonstration of the DNA repair and recombination events that accompany transformation so far reported for any member of the order Mucorales. Conclusions can only be drawn for this fungus, but the many similarities with results from previous researchers suggests that similar mechanisms are likely to occur in other Mucorales fungi. Furthermore, the recombination events identified in this study seem to follow the tenets modeled after abundant studies with other fungi, and it was anticipated that Mucorales fungi would not be an exception.

Autonomous replication of the DNA transformed into *R. oryzae* always occurred in a concatenated form of high molecular weight and unknown topology. Formation of similarly large arrangements of autonomously replicating plasmids has also been discovered in transformants of the fungi *Ustilago maydis* and *Schizosaccharomyces pombe*, and they were eventually determined to be end-to-end multimers of the transforming plasmid (Sakaguchi and Yamamoto 1982; Heyer et al. 1986; Fotheringham and Holloman 1990). However, it was not clear whether these tandem arrays were covalently closed circular arrangements, or linear structures as in *Cryptococcus neoformans*, which replicate as mini-chromosomes with telomere-like sequences (Edman 1992; Varma and Kwon-Chung 1994). The topology of transformed high-molecular-weight DNA is not known for *Mucorales* fungi and may well differ depending on whether linear or circular DNA is used for transformation. Regardless of the topology of the introduced DNA in *R. oryzae*, it appears that the copy number of multimers is quite variable. Southern analyses using restriction enzymes that do not cut the input plasmid invariably produce the most intense hybridization signals >23 kb, with lighter patterns that extend down to as low as 1 kb. Concatenates were often so large that much of the plasmid DNA was retained within the wells of the gel. Transformation of this uncleaved DNA into *E. coli* yielded plasmids that were primarily monomers and dimers (data not shown). If multimers of varying length were present in *R. oryzae* transformants, it would be expected that monomers and dimers would represent the highest percentage of plasmids recovered in *E. coli*, since larger fragments are more difficult to transform into the bacterium. None of the plasmids recovered from *R. oryzae* transformants had any indications of rearrangement or deletions, as seen with plasmids rescued from other transformed Mucorales fungi (Yanai et al. 1990, 1991; Arnau et al. 1991). However, such rearrangements are often associated with recombination in certain *E. coli* strains and may not be the result of fungal recombination.

It would be expected that, even with autonomous replication of introduced DNA, a certain percentage of circular plasmid would integrate by type-I integration events as described by Hinnen et al. (1978). This type of integration with circular plasmids has been observed in our laboratory at a low frequency of no more than 5%. Analysis of approximately 10 of these events showed that additive integration always occurred at homologous regions and occasionally resulted in the integration of several plasmid copies in tandem (data not shown). With this type of integration occurring at such a low frequency, an enrichment technique to improve the probability of isolating transformants with integrated DNA has been used with some success (Arnau et al. 1991; Arnau and Stroman 1993). This method involves growing transformants through several rounds of sporulation on non-selective medium, so that introduced DNA that is not integrated can be lost. Selection can then be used to recover isolates that still retain the selective marker.

Linearization of DNA prior to transformation was found to be the most effective way of obtaining chromosomal integration in *R. oryzae*. However, unknown repair mechanisms usually repaired these double-stranded breaks, thereby still allowing autonomous

replication of concatenated plasmids. This was evidenced by the observation that transformation with plasmid linearized at a unique restriction site (e.g., *Bam*HI or *Sty*I) resulted in the restoration of the restriction enzyme recognition site in the majority of the isolates. Religation of these types of double-stranded breaks is typically referred to as illegitimate or non-homologous end-joining, and enzyme systems that can carry out this process have previously been described in detail (Paques and Haber 1999; Lewis and Resnick 2000). However, it is misleading to portray this repair event as not involving homology, since restriction enzymes, including those used in this study, often leave compatible overhangs that can be described as micro-homology. The importance of this micro-homology was demonstrated when two different enzymes were used for linearization of the transforming DNA. Transformation with linear DNA having incompatible overhangs from *Bam*HI and *Kpn*I digestion did not yield any isolates with evidence of plasmids replicating autonomously. Instead, type-III gene replacement events occurred at the chromosomal *pyrG* locus in all of these transformants. It is unlikely that these transformants resulted from reversion of the *pyrG-181* mutation, which occurs at a frequency of $< 10^{-9}$ per spore. Furthermore, the partial deletion of the genomic *pyrG* sequence in transformant BK6 suggests a tendency for homologous recombination to occur at the next available level of homology, the *pyrG* locus in this example, when no micro-homology is present at the linearization site. This type-III gene replacement event thus represents homologous chromosomal integration and can potentially be used for disruptions of specific targets.

The addition of a 40-bp direct repeat in plasmid pPLDR reduced the tendency for the *Bam*HI to re-ligate by illegitimate end-joining; instead homologous recombination between the repeats was observed in four out of five transformants. One of these transformants, DR4, did have chromosomal integration of four copies of the transforming construct. But the loss of the *Bam*HI site on the vector sequence indicates that homologous recombination between the direct repeats occurred prior to type-I integration at the *pyrG* locus. In a similar experiment, a shift from illegitimate end-joining to homologous recombination between the direct repeats on a linearized 2 μm-containing plasmid also occurred in *S. cerevisiae* (Raymond et al. 1999).

Linearization of transforming DNA within the *pyrG* (e.g., *Sty*I digestion of pPyr225) was the most effective method for integrating the entire plasmid. Targeting integration by linearizing transforming DNA within sequences that have homology to a chromosomal locus was first demonstrated by Orr-Weaver et al. (1981, 1983) and was an important milestone for the introduction of the double-strand break repair theory as a mechanism for recombination (Szostak et al. 1983; Orr-Weaver and Szostak 1985). However, most of these early experiments were performed with non-replicating plasmids in *S. cerevisiae*, thus limiting double-stranded break repair mechanisms to integration events. Since most DNA appears to be capable of replicating autonomously in *R. oryzae*, the number of possible recombination events is greater. Even when the transforming plasmid was linearized within the *pyrG* gene, end-joining repair of the *Sty*I site occurred in at least two of the transformants, S3 and S5.

It is not clear if end-joining repair of linear plasmids represents intramolecular recombination within a single unit, intermolecular recombination between different DNA fragments, or possibly a combination of both. It is possible that continuous ligation of linearized plasmids leads to the formation of the large concatenated structures. However, the fact that no autonomously replicating DNA was found when the transforming plasmid was digested with two different enzymes suggests that intermolecular head-to-tail repair is not common. It has also been shown in this study that high-molecular-weight concatenates are present in isolates transformed with uncleaved plasmid. Therefore, it seems more likely that intramolecular repair leads to the formation of a circular plasmid monomer that is then concatenated by continuous rolling-circle replication to form a variety of different-sized multimers.

In conclusion, the recombination of transforming DNA in *R. oryzae* appears to involve the same types of double-strand break repair mechanisms that govern recombination in *S. cerevisiae*. This work demonstrates the necessity of linearizing transforming DNA in order to achieve targeted chromosomal integration, since uncut DNA replicates primarily as an autonomous structure. This work further indicates that recombination in *R. oryzae* occurs almost exclusively between regions of homology. Homology at or immediately adjacent to the linearization site seemed to have the most influence in controlling the outcome of the recombination event. Gene replacement events occurred in 20% of all transformations with linear DNA having micro-homology and exclusively when no micro-homology existed (i.e., digestion with *Bam*HI and *Kpn*I). When micro-homology existed between the single-stranded overhangs resulting from cleavage by the restriction enzyme, illegitimate end-joining of the double-strand break occurred in 40–80% of the transformants, depending on the location of cleavage. Direct repeats of only 40-bp immediately adjacent on either side of the linearization site were sufficient to shift all observed transformants to homologous recombination between the repeats. Type-I chromosomal integration of the transforming DNA was more likely to occur if the DNA adjacent to the cleavage site showed homology to a chromosomal site (i.e., *Sty*I linearized within the *pyrG*). Efficiency of integration is still considered low compared with many other industrial fungi, and accomplishing targeted homologous recombination in *R. oryzae* requires some effort to screen transformants. However, this work provides a better understanding of the recombination, repair and replication mechanisms that control the fate of transforming DNA in *R. oryzae*.

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